



Edinburgh Research Explorer

A CCR5-dependent novel mechanism for type 1 HIV gp120 induced loss of macrophage cell surface CD4

Citation for published version:

Hewson, TJ, Logie, JJ, Simmonds, P & Howie, SE 2001, 'A CCR5-dependent novel mechanism for type 1 HIV gp120 induced loss of macrophage cell surface CD4', *The Journal of Immunology*, vol. 166, no. 8, pp. 4835-42.

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

The Journal of Immunology

Publisher Rights Statement:

© 2001 by The American Association of Immunologists

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.





Measuring Cytokines? Don't Sacrifice on Quality

New Cost Effective ELISA solutions from the world leader
in immunoassays. [Learn More > RnDSystems.com/DuoSet](http://RnDSystems.com/DuoSet)



A CCR5-Dependent Novel Mechanism for Type 1 HIV gp120 Induced Loss of Macrophage Cell Surface CD4

This information is current as
of January 17, 2014.

Tim J. Hewson, James J. Logie, Peter Simmonds and Sarah
E. M. Howie

J Immunol 2001; 166:4835-4842; ;
<http://www.jimmunol.org/content/166/8/4835>

References This article **cites 52 articles**, 25 of which you can access for free at:
<http://www.jimmunol.org/content/166/8/4835.full#ref-list-1>

Subscriptions Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscriptions>

Permissions Submit copyright permission requests at:
<http://www.aai.org/ji/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/cgi/alerts/etoc>



A CCR5-Dependent Novel Mechanism for Type 1 HIV gp120 Induced Loss of Macrophage Cell Surface CD4¹

Tim J. Hewson,^{2*†} James J. Logie,^{*‡} Peter Simmonds,[†] and Sarah E. M. Howie^{*‡}

Type 1 HIV gp120 is especially effective in disrupting immune cell function because it is able to cause dysregulation of both infected and uninfected cells. We report a novel CCR5-dependent mechanism of gp120-induced CD4 loss from macrophages. An M-tropic gp120, using CCR5, is able to induce 70% loss of cell surface CD4 from macrophages within an hour. This cell surface CD4 loss is more substantial and rapid than the 20% loss observed with T-tropic gp120_{IIIB} by 3 h. The rapid and substantial CD4 loss induced by M-tropic gp120 is not observed on macrophages homozygous for the *ccr5*Δ32 mutation, which fail to express cell surface CCR5. We have used confocal imaging to show that gp120 and CD4 are internalized together by a process resembling receptor-mediated endocytosis, and that both proteins enter HLA-DR containing compartments of the macrophage. We have also shown by semiquantitative RT-PCR that, in response to CD4 loss from the cell surface, mRNA for CD4 is up-regulated and the intracellular pool of CD4 increases. CCR5 mRNA levels are also increased. It is proposed that internalization of self and viral protein and increased pools of intracellular CD4 could modulate Ag presentation efficiencies and have implications for the induction and maintenance of both productive immune responses and self-tolerance. *The Journal of Immunology*, 2001, 166: 4835–4842.

Type 1 HIV (HIV-1) causes AIDS by disrupting the anti-pathogen immune response (1). Both cell-mediated and humoral immunity are affected. Responses to recall Ags such as *Candida albicans* and tetanus toxoid (2) are lost, hypergammaglobulinaemia is seen (3) and Ab responses to the HIV-1 envelope Ag gp120 are skewed away from VH3 bearing IgGs, which are normally protective against pathogens (4). Autoimmune responses are also a feature (5, 6) including those against CD4 molecules (7–9).

HIV-1 is able to infect CD4⁺ T cells and CD4⁺ APCs, including macrophages and DC, (10–12). There is also evidence that both T cell and APC function can be compromised in uninfected cells of HIV⁺ patients (13–19). HIV-1-associated alteration of APC function has downstream consequences for the generation of adaptive immune responses. In addition, altered macrophage function has implications for the innate immune response to pathogens. In particular, lack of IL-12 production by HIV-1-infected individuals is associated with increased opportunistic infection (20, 21). Although all proteins of HIV-1 have been implicated in disrupting infected immune cell function (11, 22–25), secreted gp120 is arguably the most damaging viral product as it can also dysregulate the function of uninfected CD4⁺ cells in HIV-1-infected individuals. The structure of gp120 determines viral tropism such that most primary/macrophage-tropic isolates bind to both CD4 and CCR5 (R5-tropic gp120) while T lymphocyte-tropic variants bind to CD4 and CXCR4 chemokine receptor 4 (CXCR4) (26–29) (X4-tropic gp120).

CD4 on the surface of T lymphocytes acts as a ligand for MHC class II on the surface of APCs, which helps to stabilize their

interaction (30). CD4-MHCII binding signals through p56lck in T cells and this also prevents internalization of CD4 (31). CD4 on the surface of APCs is not associated with p56lck (32) but on both T cells and APCs CD4 acts as a receptor for the chemotactic cytokine IL-16 (33). It has been reported that gp120 can signal through CD4 and chemokine receptors to mimic the action of IL-16 and chemokines (34–36).

There have been several reports of gp120 modulating cell surface CD4 levels. Theodore et al. (37) showed that incubating gp120 with CD4⁺ T cells led to loss of surface CD4 and cellular function after 6 h, CD4 loss reached a nadir from 24 h and started to recover after 96 h. Wahl et al. (38) used naturally expressed X4-tropic gp120 from HIV-1_{IIIB} to show CD4 loss from the surface of monocytes over a period of a few days. This down-regulation of CD4 was suggested to be due to gp120-induced monocyte to macrophage differentiation. rgp120 from HIV-1_{IIIB} caused 30% loss of CD4 from in vitro-derived macrophages after 6–12 h of incubation with gp120, which was TNF-α dependent, (18, 39). Durrbaum-Landmann et al. (40) also showed that X4-tropic gp120 was able to induce a CD4 loss and a deficit in T cell stimulation in an Ag-presentation assay. Other agents, such as LPS (41) and 1,25-dihydroxy vitamin-D3 (42) have also been shown to cause CD4 loss from the macrophage cell surface.

This report compares interactions between R5- and X4-tropic gp120 and CD4⁺ monocyte-derived macrophages from noninfected healthy individuals. We present evidence for a novel mechanism of R5-tropic gp120-induced macrophage surface-CD4 loss and internalization. This loss is rapid, substantial, and does not occur with X4-tropic gp120. We have used confocal microscopy and semiquantitative RT-PCR to follow the CD4 loss from the surface and to investigate the kinetics and regulation of surface CD4 recovery.

Materials and Methods

Reagents and Abs

Unless otherwise stated, all reagents and Abs were obtained from Sigma (Poole, Dorset, U.K.).

*Immunobiology Group, Centre for Inflammation Research and [†]Laboratory for Clinical and Molecular Virology, University of Edinburgh, and [‡]Department of Pathology, University of Edinburgh Medical School, Edinburgh, United Kingdom

Received for publication June 28, 2000. Accepted for publication January 16, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants from AVERT and the Cunningham Trust.

² Address correspondence and reprint requests to Dr. Sarah Howie, Department of Pathology, University of Edinburgh Medical School, Teviot Place, Edinburgh, EH8 9AG, U.K. E-mail address: s.e.m.howie@ed.ac.uk

rHIV-1 gp120

Baculovirus-expressed rgp120 derived from the T cell line adapted virus strain HIV-1_{IIIB} (43) (GenBank accession number X01762), and baculovirus-expressed rgp120 produced from cDNA isolated from a primary macrophage of a pediatric AIDS patient (44) (GenBank accession number U72495) were obtained from the National Institute for Biological Safety and Control (London, U.K.) Centralised Facility for AIDS Reagents (South Mimms, U.K.), which is supported by European Union project European Vaccine Against AIDS (contract BMH4 97/2515), and the U.K. Medical Research Council (London, U.K.). The gene sequences used to generate both rgp120s were analyzed, assessed according to published criteria (45, 46), and confirmed to be X4-tropic and R5-tropic, respectively. The R5-tropic gp120 used in these studies has previously been shown to bind to human macrophages and inhibit *in vitro* infection by R5-tropic HIV-1 (47).

FITC conjugation of protein

Gp120 was conjugated to FITC, using a published protocol (48) and Slide-A-Lyser dialysis cassettes (Pierce and Warriner, Chester, Cheshire, U.K.). Briefly, 50 μ g gp120 was diluted to 500 μ l with distilled water and dialyzed against the labeling buffer. A total of 2 μ l of 5 μ g/ml⁻¹ FITC in DMSO was incubated with dialyzed gp120 for 2 h in the dark to allow conjugation to take place. The labeled gp120 was then dialyzed against Tris-HCl buffer to remove DMSO and unbound FITC. The FITC:gp120 ratio of the conjugate was determined using a spectrophotometer as described (48) and found to be 7:1, similar to a FITC:BSA ratio of 11.2:1 for FITC-conjugated BSA, which was used as a control protein.

Macrophage isolation and culture

Buffy coats from single, anonymous, healthy blood donations were obtained from the Scottish National Blood Transfusion Service after routine screening for the absence of Abs to HIV-1, HIV-2, hepatitis B, hepatitis C, and syphilis. Mononuclear cells (PBMNC) were isolated by centrifugation over Lymphoprep (Nycomed, Oslo, Norway) and washing in PBS. PBMNC were plated into 25-ml tissue culture flasks (Life Technologies, Paisley, U.K.) at 5×10^6 cells/ml in Iscove's medium (Life Technologies) containing antibiotics (50 IU/ml penicillin and 50 μ g/ml streptomycin; Life Technologies) and allowed to adhere to the flask for 1 h in 5% CO₂, 37°C in a humidified incubator. Nonadherent cells were removed by extensive washing and fresh medium was added (as before with the addition of 5% heat-inactivated AB normal human serum obtained from the Scottish National Blood Transfusion Service). Adherent cells were cultured at 37°C with 5% CO₂ for a further 6 days before use. At this time, the adherent cells were >95% CD14⁺, MHCII⁺, and CD4⁺ macrophages by flow cytometry.

T cell isolation and culture

Using commercially available columns (human T cell CD4 subset column kit; R&D Systems, Abingdon, U.K.), this method was used to purify CD4⁺T cells from PBMNC. The manufacturer's instructions were followed and all reagents were provided in the kit. Briefly, a PBMNC suspension was incubated with a mixture of mAbs and loaded onto the depletion column. The column contained anti-Ig-coated glass beads. B cells and CD8⁺ T cells were bound and retained in the column by F(ab')₂ interactions. Monocytes were retained by Fc interactions. The column eluate contained a highly enriched population of CD4⁺ T cells (>97% CD3⁺ and >95% CD4⁺ by flow cytometry).

Detection of CD4 by flow cytometry

To detect alterations in CD4 levels an anti-CD4 Ab, MT310, which binds to a different epitope of CD4 from that which gp120 binds to was used. Cultured macrophages were harvested from flasks by gentle scraping and resuspended at 5×10^5 cells/200 μ l in the original medium in a four-well plate (Life Technologies). In separate experiments 5×10^5 freshly isolated CD4⁺ T cells were used in place of macrophages. Rgp120, human recombinant macrophage-inflammatory protein (MIP)-1 α (NIBSC Centralised Facility for AIDS Reagents) or human rIL-16 (R&D Systems) were added to the cells at 8.3 nM (equivalent molarity to 1 μ g/ml of gp120). Cells were then incubated at 37°C for 1, 3, and 16–18 h (overnight) before preparation for flow cytometry.

Flow cytometric analysis was conducted using a Coulter Epics XL flow cytometer (Beckmann Coulter, Luton, U.K.) with a single argon ion laser operating at 488 nm. R-PE fluorescence was detected on a log scale. Relative intensities of cell surface staining were determined by comparing the mean fluorescence intensity of staining above the background staining of an isotype control between samples.

All Ab labeling steps were conducted on ice and all solutions were made up with prechilled flow-buffer (PBS, 1% BSA, 0.05% NaN₃). After incubation, cells were harvested and replated into 96-well microtiter U-bottom

plates (Life Technologies) at 1×10^5 cells/well and washed by centrifugation. The supernatant was removed and 10 μ l/900ng of R-PE-conjugated anti-CD4 (clone MT310; Dako, Cambridge, U.K.) or R-PE-conjugated isotype-matched control (IgG1) was added to each well. After incubating for 1 h on ice with the Abs, the cells were washed and resuspended in 400 μ l "flow fix" (PBS, 1% formaldehyde) in tubes for analysis. A total of 10,000 events were counted from each sample. Means and SEs were calculated for each treatment and means were compared using an unpaired Mann-Whitney *U* test.

Dose response of CD4 loss

Under similar conditions to those described above, R5-tropic gp120 was incubated at a range of doubling concentrations from 1/16–4 μ g/ml with 5×10^5 *ccr5* wild-type macrophages for 3 h before having CD4 levels assayed as described above.

Demonstration of competitive and noncompetitive binding

To demonstrate that neither R5-tropic gp120 or gp120_{IIIB} competes for CD4 binding with MT310, the mAb used to assay cell surface CD4 levels, the following control was undertaken. A total of 5×10^5 *ccr5* wild-type macrophages were incubated for 1 h on ice with 1 μ g/ml of either one of the gp120s used in this paper in 100 μ l of flow buffer. Cells were then washed once in flow buffer and stained for surface CD4 using clone MT310 mAb as described above, or QS4120 anti-CD4 (Sigma), before analysis by flow cytometry.

Detection of surface-bound gp120

Six-day-old *ccr5* wild-type macrophages were harvested from culture flasks and placed at 10^5 cells per well in a round-bottom 96-well plate, washed twice with flow buffer and pelleted. A total of 10 μ l R5-tropic gp120 or gp120_{IIIB} at 1 μ g/ml⁻¹ in flow buffer was added to the cell pellet, which was then agitated and incubated on ice for 2 h. After washing four times with ice-cold flow buffer, 10 μ l of 0.5 μ g/ml⁻¹ polyclonal sheep anti-gp120 serum was added to the cell pellets (ARP0734; NIBSC Centralised Facility for AIDS Reagents). Cells were incubated on ice for a further 2 h before a single wash in flow buffer, the addition of 10 μ l of 10 μ g/ml⁻¹ biotinylated donkey anti-sheep serum (Sigma) and incubation on ice for 1 h. Following another wash, 10 μ l of 1 μ g/ml⁻¹ R-PE-conjugated streptavidin (Sigma) was added to each well and incubated for 1 h on ice. Cells were then washed twice in flow buffer and suspended in flow fix, as detailed above, for analysis by flow cytometry.

Staining of macrophages for fluorescence microscopy

Monocyte-derived human macrophages were grown from buffy coats as above, except that 5×10^6 cells in 1 ml medium were plated onto 70% ethanol-sterilized glass coverslips (BDH, Poole, Dorset, U.K.) in the bottom of 6-well plates (Life Technologies). After 6 days, cells were incubated with FITC-gp120 or FITC-BSA for various lengths of time. Cells were then fixed by removal of medium and the addition of 1 ml of 2% paraformaldehyde in PBS to each well for 20 min at room temperature.

After fixation, the coverslips were washed three times in PBS. Cells were permeabilized by the addition of 1 ml/well of 0.1% Triton X-100 in PBS for 5 min at room temperature, washed three times in PBS, and blocked with 1 ml/well of 0.2% BSA in PBS at room temperature for 10 min. Cells were stained with FITC-conjugated anti-CD4 (1.8 μ g/ml⁻¹, MT310; Dako), nonconjugated anti-HLA-DR (3 μ g/ml⁻¹, DA6 231; a gift from Keith Guy (Department of Biological Science, Napier University, Edinburgh, U.K.)) followed by TRITC-conjugated goat anti-mouse IgG and then FITC-conjugated anti-CD4, or with nonconjugated CD4 (1.8 μ g/ml⁻¹, MT310; Dako) followed by TRITC-conjugated goat anti-mouse IgG. Abs were diluted in 1% BSA, 0.05% NaN₃ in PBS. Each Ab was added for 30 min at room temperature followed by three PBS washes. A 100- μ l drop of the diluted Ab was added to a piece of Parafilm (Merck, Poole, Dorset, U.K.), which was placed on a damp paper tissue in a plastic tray; the coverslip to be stained was then carefully lowered, taking care to avoid trapping air bubbles, onto the drop with the cell side facing down. After incubation, the coverslip was gently floated off the Parafilm with PBS. After staining coverslips were washed three times with distilled water, dried, and mounted onto glass slides (BDH) using Vector-shield anti-photo-bleaching glycerol-based mounting medium (Vector Laboratories, Orton Southgate, Cambridgeshire, U.K.). The edges of the coverslips were sealed with nail varnish and the slides were stored in the dark at 4°C for up to 2 wk before examination.

Laser-scanning confocal microscopy and image analysis

Stained macrophages were visualized on a DMRE laser scanning confocal microscope with a TCS NT image capture computer system (Leica Microsystems, Heidelberg, Germany). Images were saved as tagged image files and analyzed by the TCS NT system and Scion Image (Scion, Frederick, MD).

Macrophage *ccr5* Genotyping by RT-PCR

Total RNA was extracted from freshly isolated PBMC using the Qiagen RNeasy spin column kit as per the manufacturers instructions (Qiagen, Crawley, U.K.). RNA content was measured using a GeneQuant photo-spectrometer (Pharmacia Biotech, St. Albans, U.K.).

A total of 0.1 μ g RNA was used for cDNA synthesis using the Expand reverse transcriptase kit (Boehringer Mannheim, Roche Diagnostic Systems, Lewes, U.K.) and oligo(dT) (Oswel, Southampton, U.K.). Products of this reaction were used as templates for PCR amplification using *Taq*-Supreme DNA polymerase (Helena Bioscience, Sunderland, U.K.) and primers (Oswel) spanning the $\Delta 32$ deletion site in the *ccr5* gene or a portion of the housekeeping gene β -actin. Primer sequences were: *ccr5*: antisense, CCT GTG CCT CTT CTC ATT TCG, sense, CAA AAA GAA GGT CTT CAT TAC ACC; β -actin: antisense, CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG, sense, TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA. The thermal cycler program consisted of 5 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s; then 72°C for 10 min.

PCR products were run on a 3% agarose gel with appropriate m.w. markers (pGEM; Promega, Southampton, U.K.). Bands were visualized by ethidium bromide staining and UV *trans*-illumination. Donors were *ccr5* genotyped based on PCR fragment size (see Fig. 1 for example).

Semiquantitative RT-PCR

RNA was extracted and assayed, and 0.1 μ g was reverse transcribed from samples as described above. A variable cycle number PCR was conducted to determine the optimum number of cycles required for a near-linear relationship between the RNA level and resultant DNA band intensity. All cDNAs were then amplified by PCR for this number of cycles and the band intensities following ethidium bromide staining and *trans*-illumination was determined by the Enhanced Analysis System (EASY, version 4.19; Scotlab, Coatbridge, Lanarkshire, U.K.). To allow for errors in the initial equalization of RNA mass between samples, samples were compared as ratios of the intensity of a band amplified from the gene of interest to the intensity of a housekeeping gene band from the same cDNA amplified by the same thermal cycler program (47). *CD4* and *ccr5* message levels were compared thus using the following programs and primers: *CD4*: sense, GCA GTG CGG AGC TGT GGT, anti-sense, GGG TCC CCA CAC CTC ACA GG; and *ccr5* and β -actin primers as above. The *CD4* thermal cycler program consisted of 32 cycles of 94°C for 1 min, 58°C for 2 min, and 72°C for 2

min. The *Ccr5* thermal cycler program consisted of 5 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; then 29 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s.

Results

CCR5-dependent loss of cell surface CD4 is induced by R5 tropic gp120

All buffy coat donors were screened for the *ccr5* $\Delta 32$ mutation (Fig. 1), of 67 samples 52 were found to be homozygous wild type, 10

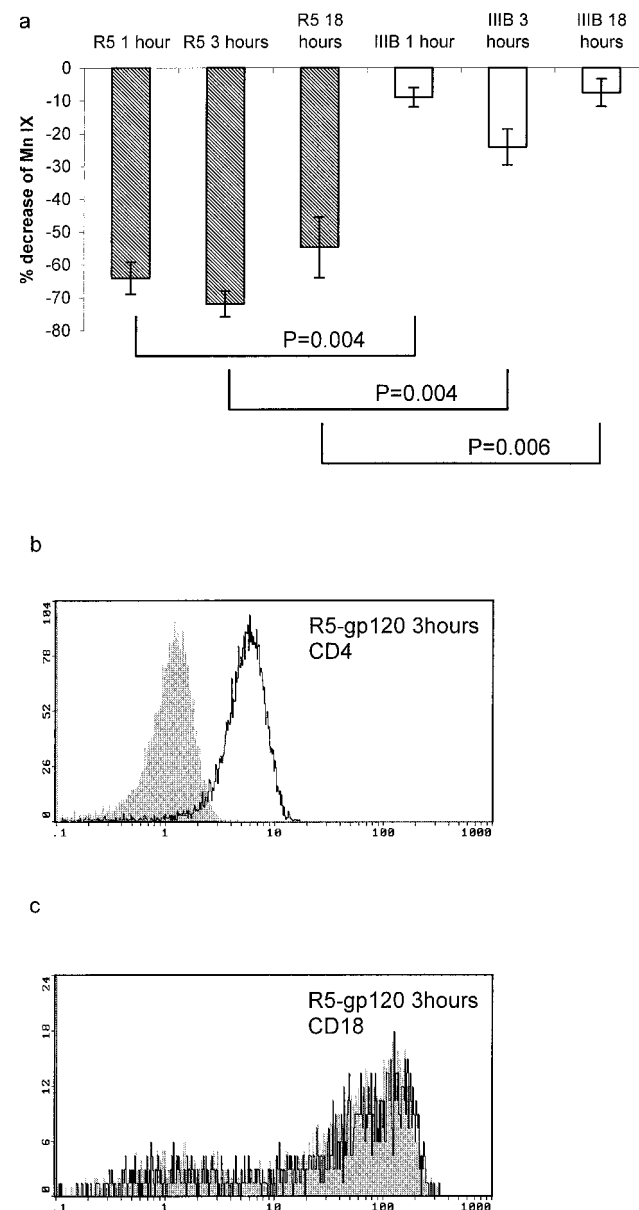


FIGURE 2. The effect of gp120s on the cell surface CD4 level of *ccr5* $\Delta 32$ wild-type macrophages. Macrophages were incubated with R5-tropic gp120 or gp120_{IIIB} for the time intervals indicated. The normalized MnIX values are calculated from the mean fluorescence intensity of the anti-CD4 labeled cells. The MnIX value above the background fluorescence obtained with the isotype control is then rescaled to be expressed as a percentage decrease of the initial (untreated) staining intensity. The graph (a) shows the mean data values obtained from six different donors. The bars show SEM and the *p* values were calculated using an unpaired Mann Whitney *U* test. Raw data showing CD4 (b) and CD18 (c) levels before (black line) and after (solid gray) 3 h of incubation with R5-tropic gp120 in wild-type macrophages showing a typical response is also given.

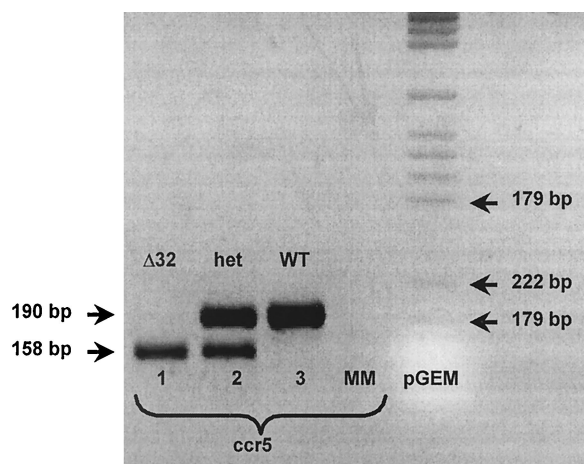


FIGURE 1. Genotyping gel. RNA extracted from buffy coats 1, 2, and 3 was subjected to RT-PCR for *ccr5*. The products show a length polymorphism with wild-type message amplifying to a 190-bp product and the $\Delta 32$ mutant message amplifying to a 158-bp product. This allows sample 1 to be detected as homozygous for the $\Delta 32$ mutation. Sample 2 is heterozygous, and sample 3 is homozygous wild type.

Table I. R5-tropic, but not X4-tropic, gp120 induces macrophage CD4 loss

Length of Treatment (h)	MnIX Relative to 100% Starting Value			
	R5-gp120	gp120 _{IIIB}	MIP-1 α	IL-16
0	100	100	100	100
1	35	78	89	97
3	32	102	92	88
18	34	97	80	76

were heterozygous, and 5 were homozygous mutant. Using wild-type donors' macrophages, 1 μ g/ml gp120_{IIIB} induces a maximal 12–25% CD4 loss at 3 h, which does not reach statistical significance. A total of 1 μ g/ml R5-tropic gp120 induced substantial and significant (65–75%) loss of cell surface CD4 at both 1 and 3 h. After 18 h, the loss was still significant, while CD4 levels had started to recover (Fig. 2). Levels of a non-gp120-binding cell surface marker, CD18, remained unchanged (Fig. 2). Although R5-tropic gp120 caused a substantial loss of surface CD4, the CD4 ligand, IL-16, and the CCR5 ligand, MIP-1 α , had no effect (Table I).

To demonstrate that both gp120s were able to bind to macrophage cell surfaces, gp120 was allowed to bind to *ccr5* wild-type macrophages and was then detected by an anti-gp120 Ab (Fig. 3). To study the dose dependency of surface CD4 loss, both gp120s were incubated separately at a range of concentrations for 3 h with *ccr5* wild-type macrophages. Table II shows that changes in CD4 level are not greatly influenced by gp120 dose (as opposed to coreceptor usage) over the concentrations examined. To demonstrate that apparent cell surface CD4 loss was not simply due to R5-tropic gp120 competing with anti-CD4 (clone MT310) for CD4 binding, both gp120s were separately incubated with *ccr5* wild-

Table II. R5-tropic gp120 induces macrophage CD4 loss at low concentration

Concentration (μ g/ml)	MnIX Relative to 100% Starting Value			
	Donor 1		Donor 2	
	R5-gp120	gp120 _{IIIB}	R5-gp120	gp120 _{IIIB}
Nil	100	100	100	100
0.0625	40	92	35	80
0.125	41	104	32	78
0.25	38	92	34	82
0.5	37	98	38	79
1	35	84	32	76
2	30	84	31	81
4	31	83	29	77

Table III. gp120 binding does not compete with MT310 anti-CD4 Ab

Anti-CD4 Clone Used for Detection	MnIX Relative to 100% Value Obtained Without Preincubation					
	Donor 1			Donor 2		
	Nil	R5-gp120 (1 μ g/ml)	gp120 _{IIIB} (1 μ g/ml)	Nil	R5-gp120 (1 μ g/ml)	gp120 _{IIIB} (1 μ g/ml)
MT310	100	92	102	100	104	98
QS4120	100	28	24	100	27	26

type macrophages at 4°C in the presence of NaN₃ (to prevent endocytosis). This treatment did not result in a reduced ability of anti-CD4 Ab MT310 to bind to CD4 (Table III). However, binding of the anti-CD4 monoclonal QS4120, previously shown to bind to the gp120 binding site of CD4 (49), was inhibited by preincubation with gp120 (Table III).

To confirm that this effect was dependent on intact CCR5 receptor, the same experiment was repeated on macrophages from CCR5 Δ 32 mutant donors and neither gp120_{IIIB} or R5-tropic gp120 was able to induce a significant cell surface CD4 loss (Table IV).

Loss of surface CD4 is accompanied by increased levels of CD4 in an internal HLA-DR-positive pool

Ccr5 wild-type macrophages were permeabilized and stained for CD4 after various periods of incubation with R5-tropic gp120. Cell surface CD4 declines after incubation with R5-tropic gp120. Intracellular pools of CD4, indicated by cytoplasmic staining (Fig. 4) increase after gp120 incubation.

Fig. 4 shows a coincidence of intracellular staining for CD4 and HLA-DR in the cytoplasm of macrophages that have been incubated with R5-tropic gp120. This suggests that the intracellular CD4 staining seen in Fig. 6 is localized to the endoplasmic reticulum.

Table IV. R5-tropic gp120-induced CD4 loss is dependent on the presence of CCR5

Length of Treatment (h)	MnIX Relative to 100% Starting Value			
	Donor 1		Donor 2	
	R5-gp120	gp120 _{IIIB}	R5-gp120	gp120 _{IIIB}
0	100	100	100	100
1	92	88	116	105
3	98	103	99	86
18	109	113	101	101

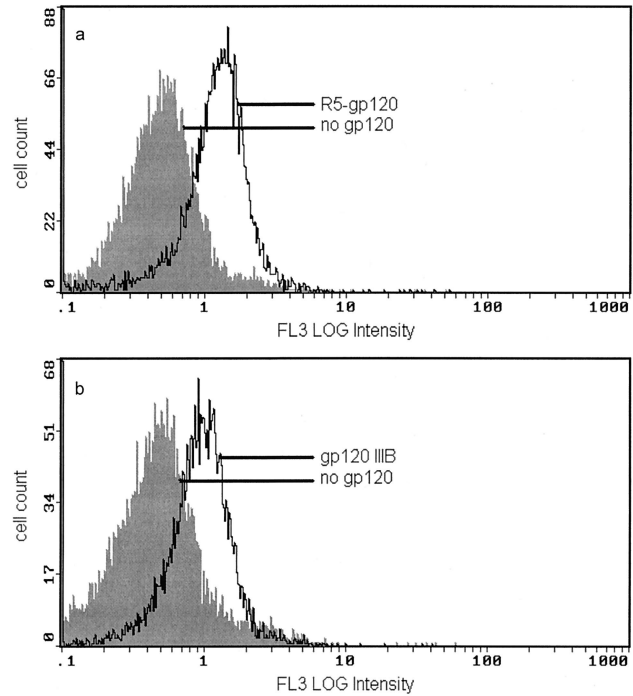


FIGURE 3. Detection of macrophage surface-bound gp120. Macrophages were incubated with R5-tropic gp120 (a) or gp120_{IIIB} (b). The surface-bound gp120s were then detected by flow cytometry using an anti-gp120 polyclonal Ab, which was linked to FITC-conjugated streptavidin. Incubation of cells with gp120 resulted in a higher fluorescence signal than cells stained with anti-gp120 in the absence of gp120.

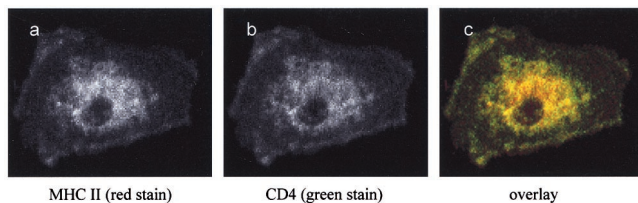


FIGURE 4. Confocal micrographs of a macrophage that has been incubated with R5-tropic gp120 for 3 h and then fixed, permeabilized, and stained red for MHC class II (a) and green for CD4 (b). CD4 and MHC are colocalized into the same intracellular compartments (these will include the endoplasmic reticulum and the Golgi apparatus) as indicated by the coincidence of the red and green stains to give a yellow stain (c).

R5-tropic gp120 is endocytosed by ccr5-wild-type macrophages in a way distinct from other proteins

Macrophages were stained red for HLA-DR at various time-points after being exposed to FITC-conjugated (green) R5-tropic gp120 or BSA (Data for gp120_{IIIB} is not shown but is similar to that for BSA). After 20–60 min (Fig. 5c) BSA enters the macrophages as numerous, small endocytic vesicles, these persist for many hours but by 2 h there is evidence of breakdown of the BSA-FITC conjugate in the form of a more diffuse green stain. In contrast, R5-tropic gp120 enters as one or two larger and much more brightly staining vesicles from 20 min (Fig. 5b), smaller vesicles only become visible from ~1–2 h (visible at 18 h, Fig. 5d). There is evidence of gp120-FITC conjugate breakdown from 2–3 h. Both proteins are detected in HLA-DR containing compartments (at 3–4 h for BSA and at 2 h for R5-tropic gp120).

R5-tropic gp120 enters cells along with CD4, gp120_{IIIB} and BSA does not

A few large vesicles of R5-tropic gp120 appear in macrophages after 20 min of incubation and these also contain CD4 (Fig. 6a).

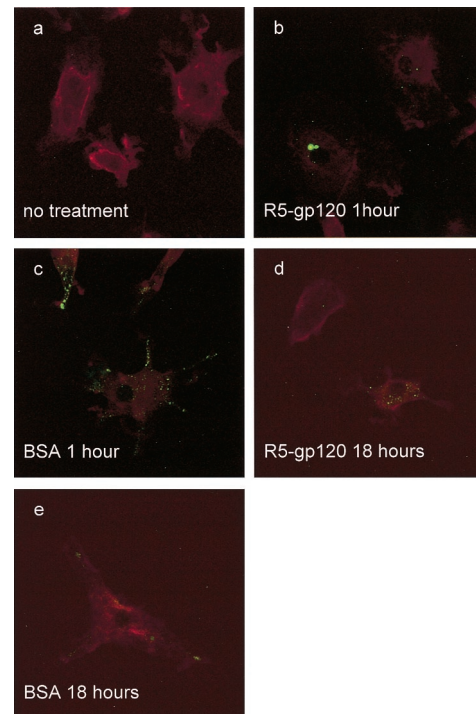


FIGURE 5. Confocal micrographs showing macrophages that have been incubated with FITC-conjugated R5-tropic gp120 or a control FITC-conjugated protein (BSA) for the times indicated then fixed, permeabilized, and stained red for MHC class II. Internalized protein is visible in green.

The vesicles containing endocytosed X4-tropic gp120_{IIIB} (Fig. 6b) or BSA (Fig. 6c) do not contain CD4 at any time-point studied and are smaller, less brightly stained, and more numerous. Such characteristics resemble the appearance of pinocytotic vesicles. Cell surface CD4 staining is less diminished and more visible in Fig. 5

FIGURE 6. Confocal micrographs showing macrophages that have been incubated with FITC-conjugated R5-tropic gp120 (a), gp120_{IIIB} (b) or a control FITC-conjugated protein (BSA, c) for 20 min then fixed, permeabilized, and stained red for CD4. Internalized protein is visible in green. Coincidence of the red and green stains gives a yellow stain. The coincidence of the two stains in a, but not in b or c, is confirmed by software analysis of the image and shown by the resultant intensity plot, which shows the intensity of red and green staining along the line drawn through the image of the cell as indicated

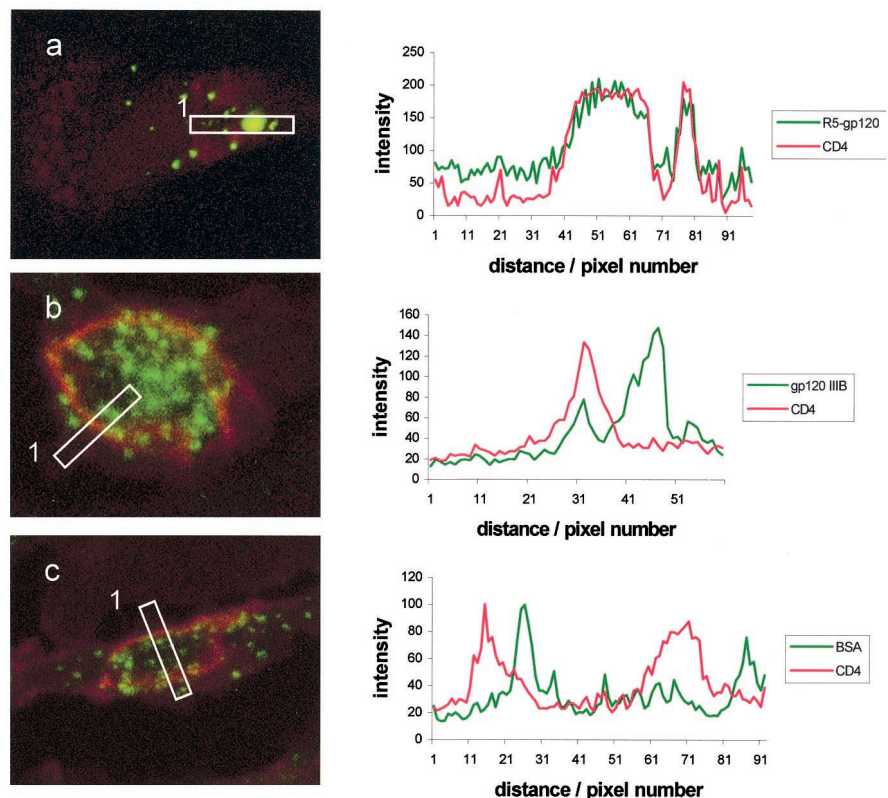
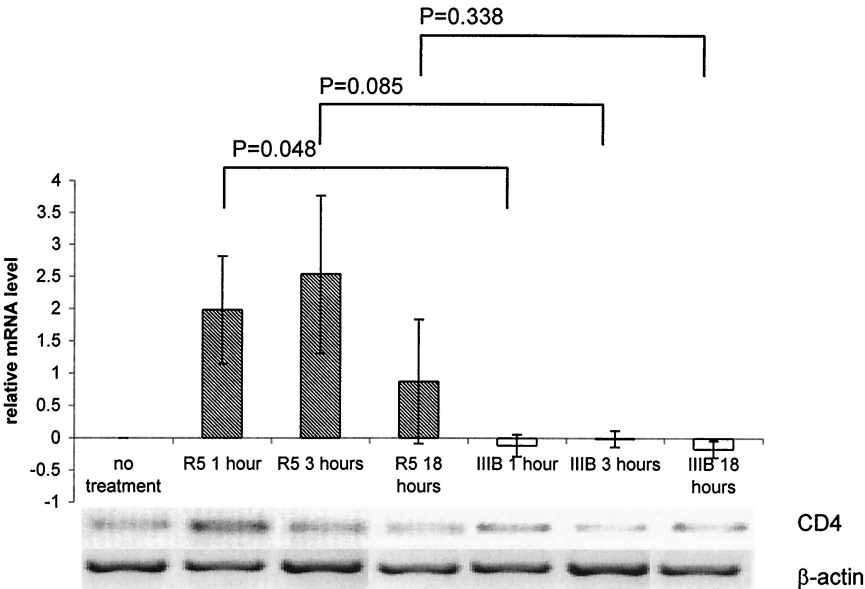


FIGURE 7. A graph to show the ratio of CD4 cDNA to β -actin cDNA expressed relative to the starting value of 1. Macrophages were incubated for the times indicted with R5-tropic gp120 or gp120_{IIIB} before RNA harvest. The graph shows the mean data values obtained from six different donors. The bars show SEM and the *p* values were calculated using an unpaired Mann Whitney *U* test. The gel picture underneath graph shows raw data from one of the six donors.



after incubation with gp120_{IIIB} (Fig. 6b) or BSA (Fig. 6c) than after incubation with R5-tropic gp120 (Fig. 6a)

R5 tropic gp120 induces an up-regulation of mRNA for CCR5 and CD4

Figs. 7 and 8 show that R5-tropic gp120 but not X4-tropic gp120 induces a concomitant up-regulation in CD4 and CCR5 transcript. The increased mRNA is visible from 1 h and persists until ~18 h (overnight) when levels start to fall again.

Surface CD4 loss does not occur on T cells

Incubating with either M- or T-tropic gp120 does not cause a loss of surface CD4 from CD4⁺ T cells (Table V).

Discussion

It has been reported that HIV-1 gp120 is able to disrupt the function of immune cells (1, 11, 50) and that one of its actions is to cause a loss of cell surface CD4 and the functions associated with

this protein (18, 37, 38, 40). Our evidence identifies a novel, CCR5-dependent mechanism of CD4 decline.

We observed a relatively small (~25%) surface CD4 decline induced on macrophages by gp120_{IIIB} (Fig. 2) This X4-tropic gp120_{IIIB}-induced response may be similar to the CD4 loss reported by Wahl et al. (38) and Karsten et al. (18). Although CXCR4 is expressed on human macrophages and can, in some circumstances, be used for HIV-1 entry, it appears to be unusable and possibly inaccessible to many T cell line adapted X4-tropic gp120s including gp120_{IIIB}, which was used in this study as a prototypic X4-tropic gp120 (51).

When R5-tropic gp120 was used, we observed a cell surface CD4 loss that was more substantial than both previous reports and our observations with X4-tropic gp120_{IIIB}. Approximately 75% of the surface CD4 was lost by 3 h after the addition of the gp120. This observation requires that a novel mechanism of CD4 loss be proposed. Further evidence implicates CCR5 binding, in addition

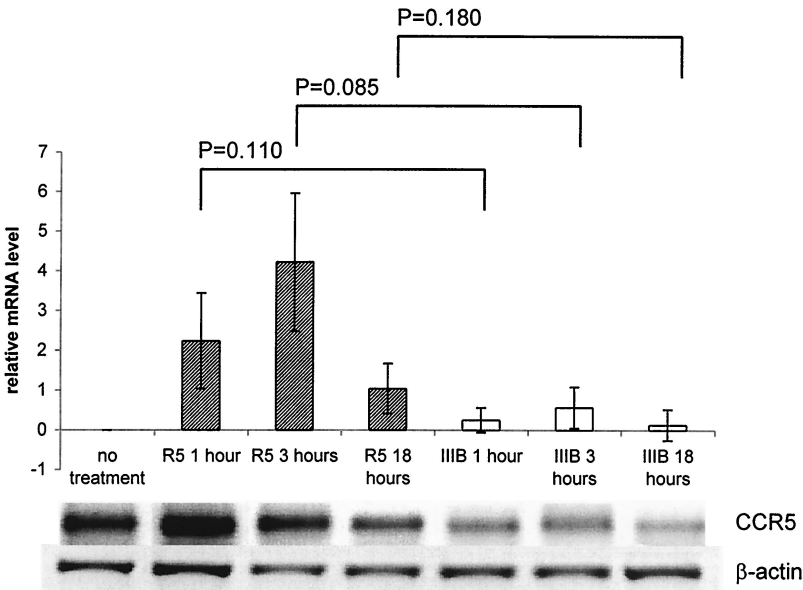


FIGURE 8. A graph to show the ratio of CCR5 cDNA to β -actin cDNA expressed relative to the starting value of 1. Macrophages were incubated for the times indicted with R5- or X4-tropic gp120 before RNA harvest. The graph shows the mean data values obtained from six different donors. The bars show SEM and the *p* values were calculated using an unpaired Mann Whitney *U* test. The gel picture underneath graph shows raw data from one of the six donors.

Table V. *gp120 binding does not down-regulate CD4 on T cells*

Length of Treatment (h)	MnIX relative to 100% starting value			
	Donor 1		Donor 2	
	R5-gp120	gp120 _{IIIB}	R5-gp120	gp120 _{IIIB}
0	100	100	100	100
1	96	108	79	102
3	100	86	98	90
18	90	83	74	88

to CD4 binding, as a requirement for the operation of this novel mechanism.

The requirement for gp120 to bind to CCR5 to obtain a substantial CD4 loss is suggested by the strain specificity of the effect, with substantial loss only observed when R5-tropic gp120 is used. Further evidence for a CCR5 binding requirement comes from the observation that CD4 loss is not observed in mutant macrophages, which do not express CCR5 (Table II). Binding to CD4 but not CCR5 (by gp120_{IIIB}, IL-16, or on *ccr5*-null cells) or binding to CCR5 only (by MIP-1 α) does not induce substantial CD4 loss (Table I).

Therefore, substantial CD4 loss by this newly described mechanism requires gp120 to bind to both CD4 and CCR5. We propose that the CD4 loss observed be due to cross-linking of CD4 and CCR5 on the macrophage cell surface followed by endocytosis of the tri-protein complex. Single ligation of CD4 or CCR5 does not produce sufficient cross-linking to allow endocytosis and CD4 loss by this mechanism. An assumption of this model is that it is the same molecule of gp120 that binds to both cell surface receptors. CXCR4 on the macrophage appears to be inaccessible to X4-tropic gp120_{IIIB} binding in this way; whereas, CCR5 is present on the macrophage cell surface in a form that allows R5-tropic gp120 to bind to both it and CD4. It may be that CD4 and CCR5 are pre-associated in some way on the macrophage cell surface (52, 53) and that this allows molecules of R5-tropic gp120 to bind to these surface molecules and produce the CD4 loss demonstrated. Such a preassociation could explain the apparent selection by HIV-1 of CCR5 for almost exclusive use as a coreceptor for macrophage infection. Alternatively CCR5 and CD4 ligation may be required to cause macrophage specific intracellular signaling events required for the induction of endocytosis. If this were the case and receptor cross-linking was not also a requirement for substantial CD4 loss, one would expect to see macrophage surface CD4 loss induced by coinubation with gp120_{IIIB} and MIP-1 α . Preliminary experiments (data not shown) fail to show this.

The analogous in vivo situation may be more complicated. Gp120 has been found at high levels in the serum of AIDS and AIDS-related complex patients (54). Serum Abs to gp120 may increase the extent of macrophage surface receptor cross-linking, bring the Fc receptor into play, and reduce the CCR5-binding requirement. It has been observed that Abs to gp120 can enhance cell infection (55) by increasing virion-to-cell binding.

Our model of cross-linking-induced endocytosis is strengthened by the confocal images obtained. Figs. 4 and 5 show that R5-tropic gp120 enters macrophages in a form that is different, in terms of size and number of vesicle and localization with CD4, from pinocytosis of BSA, a protein with no specific cell surface receptor, and endocytosis of gp120_{IIIB}, a protein that is unable to use both a cell surface receptor and a coreceptor. R5-tropic gp120 enters cells as a few large vesicles, possibly by a process of capping. Endocytosed gp120_{IIIB} and BSA appears in macrophages in a form that suggests that it entered by a process more akin to pinocytosis. Loss

of surface CD4 from CD4⁺ T cells could not be induced by either gp120. One might have expected X4-tropic gp120_{IIIB} to cause a loss of T cell surface CD4 by cross-linking CD4 and CXCR4. It may be that despite gp120_{IIIB} producing TCR cross-linking, T cells lack the cellular machinery for phagocytosis that is present in macrophages.

If our proposed model of cross-linking-induced endocytosis operates in vivo, there are implications for processing and presentation of large amounts of both viral and self-protein entering an APC. The induction of protective and autoimmune responses could be influenced if the endocytosed proteins were finding their way into Ag-presentation pathways and being presented with unusually high efficiency.

Figs. 4, 7, and 8 show that after R5-tropic gp120-induced cell surface loss of CD4 (and presumably CCR5 also), the cellular production of these proteins is stepped up. This is manifested as an up-regulation of mRNA transcript (Figs. 6 and 7) and, certainly in the case of CD4 (Fig. 3) at the level of increased translation of mRNA into intracellular pools of protein. Increased levels of intracellular self-protein must have in vivo implications for the possible breaking of tolerance and the induction of autoimmune responses to these proteins.

Acknowledgments

We thank Marilyn Moore and Donald Innes who initiated the macrophage isolation and culture methods for their assistance in setting up this project; and the Edinburgh center of the Scottish National Blood Transfusion Service for generous provision of buffy coats and human serum. Invaluable technical assistance in the capture of confocal images was provided by Linda Sharp (Department of Biomedical Sciences, University of Edinburgh).

References

1. Rodriguez, G. E., and R. C. Hard. 1995. Immunopathogenesis of AIDS. *Immunol. Allergy Clin. N. Am.* 15:225.

2. Tassinari, P., L. Deibis, I. Blanca, N. E. Bianco, and G. E. Deperez. 1995. Decreased T-cell proliferative response to common environmental antigens could be an indicator of early human immunodeficiency virus-mediated lymphocyte lesions. *Clin. Diag. Lab. Immunol.* 2:404.

3. Coyle, T. E. 1997. Hematologic complications of human immunodeficiency virus infection and the acquired immunodeficiency syndrome. *Med. Clin. North. Am.* 81:449.

4. Juompan, L., P. Lambin, and M. Zouali. 1998. Selective deficit in antibodies specific for the superantigen binding site of gp120 in HIV infection. *FASEB J.* 12:1473.

5. Stricker, K., E. Knipping, T. Böhler, A. Benner, P. H. Krammer, and K. M. Debatin. 1998. Anti-CD95 (APO-1/Fas) autoantibodies and T cell depletion in human immunodeficiency virus type 1 (HIV-1)-infected children. *Cell Death Differ.* 5:222.

6. Marchalonis, J. J., N. M. Ampel, S. F. Schluter, A. Garza, D. F. Lake, J. N. Gagliani, and W. J. Landsperger. 1997. Analysis of autoantibodies to T-cell receptors among HIV-infected individuals: epitope analysis and time course. *Clin. Immunol. Immunopathol.* 82:174.

7. Furci, L., A. Beretta, A. Siccardi, A. Lazzarin, C. Confetti, Z. Magnani, P. Scarpellini, L. Lopalco, and S. E. Burastero. 1997. Human immunodeficiency virus type 1 glycoprotein 120-specific T lymphocytes provide intermolecular help for anti-CD4 autoantibody production in exposed uninfected subjects. *AIDS Res. Hum. Retroviruses* 13:1461.

8. Gessani, S., P. Puddu, B. Varano, P. Borghi, L. Conti, L. Fantuzzi, and F. Belardelli. 1994. Induction of β -interferon by human-immunodeficiency-virus type-1 and its gp120 protein in human monocytes-macrophages: role of β -interferon in restriction of virus-replication. *J. Virol.* 68:1983.

9. Caporossi, A. P., G. Bruno, S. Salemi, C. Mastroianni, M. Falciano, A. Salotti, N. Bergami, I. Santilio, R. Nisini, and V. Barnaba. 1998. Autoimmune T-cell response to the CD4 molecule in HIV-infected patients. *Viral Immunol.* 11:9.

10. Barré-Sinoussi, F. 1988. HIV target-cells: effect of their infection by HIV on the pathogenesis of AIDS. *Lymphology* 21:11.

11. Hewson, T., N. Lone, M. Moore, and S. Howie. 1999. Interactions of HIV-1 with antigen-presenting cells. *Immunol. Cell Biol.* 77:289.

12. Fidler, S. J., and A. D. M. Rees. 1999. Antigen presenting cell function in HIV-1-infected patients. *Immunol. Lett.* 66:129.

13. Chirmule, N., and S. Pahwa. 1996. Envelope glycoproteins of human immunodeficiency virus type 1: Profound influences on immune functions. *Microbiol. Rev.* 60:386.

14. Desbarats, J., J. H. Freed, P. A. Campbell, and M. K. Newell. 1996. Fas (CD95) expression and death-mediating function are induced by CD4 cross-linking on CD4⁺ T cells. *Proc. Nat. Acad. Sci. USA* 93:11014.
15. Heinkelein, M., M. Muller, O. Kutsch, S. Sopfer, and C. Jassoy. 1997. Rapid and selective depletion of CD4⁺ T lymphocytes and preferential loss of memory cells on interaction of mononuclear cells with HIV-1 glycoprotein-expressing cells. *J. AIDS Hum. Retrovirol.* 16:74.
16. Hewson, T. J., and S. E. M. Howie. 1998. The Effects of HIV gp120 on the expression of antigen presenting cell (APC) surface molecules. *Immunology* 95:86.
17. Kaneko, H., T. Hishikawa, I. Sekigawa, H. Hashimoto, K. Okumura, and Y. Kaneko. 1997. Role of tumour necrosis factor- α (TNF- α) in the induction of HIV-1 gp120-mediated CD4⁺ T cell anergy. *Clin. Exp. Immunol.* 109:41.
18. Karsten, V., S. Gordon, A. Kirn, and G. Herbein. 1996. HIV-1 envelope glycoprotein gp120 down-regulates CD4 expression in primary human macrophages through induction of endogenous tumour necrosis factor- α . *Immunology* 88:55.
19. Wagner, R. P., S. M. Levitz, A. Tabuni, and H. Kornfeld. 1992. HIV-1 envelope protein (gp120) Inhibits the activity of human bronchoalveolar macrophages against *Cryptococcus neoformans*. *Am. Rev. Respir. Dis.* 146:1434.
20. Chehimi, J., and G. Trinchieri. 1994. Interleukin-12: a bridge between innate resistance and adaptive immunity with a role in infection and acquired immunodeficiency. *J. Clin. Immunol.* 14:149.
21. Marshall, J. D., J. Chehimi, G. Gri, J. R. Kostman, L. J. Montaner, and G. Trinchieri. 1999. The interleukin-12-mediated pathway of immune events is dysfunctional in human immunodeficiency virus-infected individuals. *Blood* 94:1003.
22. De, S. K., C. N. S. Venkateshan, P. Seth, D. C. Gajdusek, and C. J. Gibbs. 1998. Adenovirus-mediated human immunodeficiency virus-1 Nef expression in human monocytes/macrophages and effect of Nef on downmodulation of Fc γ receptors and expression of monokines. *Blood* 91:2108.
23. Peter, F. 1998. HIV nef: the mother of all evil? *Immunity* 9:433.
24. Rhee, S. S., and J. W. Marsh. 1994. Human-immunodeficiency-virus type-1 nef-induced down-modulation of CD4 is due to rapid internalization and degradation of surface CD4. *J. Virol.* 68:5156.
25. Willey, R. L., F. Maldarelli, M. A. Martin, and K. Strebel. 1992. Human-immunodeficiency-virus type-1 vpu protein induces rapid degradation of CD4. *J. Virol.* 66:7193.
26. Rizzuto, C. D., R. Wyatt, N. Hernandez-Ramos, Y. Sun, P. D. Kwong, W. A. Hendrickson, and J. Sodroski. 1998. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. *Science* 280:1949.
27. Hoffman, T. L., and R. W. Doms. 1999. HIV-1 envelope determinants for cell tropism and chemokine receptor use. *Mol. Membr. Biol.* 16:57.
28. Choe, H. 1998. Chemokine receptors in HIV-1 and SIV infection. *Arch. Pharm. Res.* 21:634.
29. Wu, L. J., N. P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A. A. Cardoso, E. Desjardins, W. Newman, et al. 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* 384:179.
30. Sakihama, T., A. Smolyar, and E. L. Reinherz. 1995. Oligomerization of CD4 is required for stable binding to class-II major histocompatibility complex proteins but not for interaction with human-immunodeficiency-virus gp120. *Proc. Nat. Acad. Sci. USA* 92:6444.
31. Pelchen-Matthews, A., I. Boulet, D. R. Littman, R. Fagard, and M. Marsh. 1992. The protein tyrosine kinase p56lck inhibits CD4 endocytosis by preventing entry of CD4 into coated pits. *J. Cell Biol.* 117:279.
32. Bowers, K., C. Pitcher, and M. Marsh. 1997. CD4: a co-receptor in the immune response and HIV infection. *Int. J. Biochem. Cell Biol.* 29:871.
33. Center, D. M., H. Kornfeld, and W. W. Cruikshank. 1996. Interleukin 16 and its function as a CD4 ligand. *Immunol. Today* 17:476.
34. Iyengar, S., D. H. Schwartz, and J. E. K. Hildreth. 1999. T cell-tropic HIV gp120 mediates CD4 and CD8 cell chemotaxis through CXCR4 independent of CD4: implications for HIV pathogenesis. *J. Immunol.* 162:6263.
35. Misse, D., M. Cerutti, N. Noraz, P. Jourdan, J. Favero, G. Devauchelle, H. Yssel, N. Taylor, and F. Veas. 1999. A CD4-independent interaction of human immunodeficiency virus-1 gp120 with CXCR4 induces their cointernalization, cell signaling, and T-cell chemotaxis. *Blood* 93:2454.
36. Theodore, A. C., D. M. Center, J. Nicoll, G. Fine, H. Kornfeld, and W. W. Cruikshank. 1996. CD4 ligand IL-16 inhibits the mixed lymphocyte reaction. *J. Immunol.* 157:1958.
37. Theodore, A. C., H. Kornfeld, R. P. Wallace, and W. W. Cruikshank. 1994. CD4 modulation of noninfected human T-lymphocytes by HIV-1 envelope glycoprotein gp120: contribution to the immunosuppression seen in HIV-1 infection by induction of CD4 and CD3 unresponsiveness. *J. AIDS Hum. Retrovirol.* 7:899.
38. Wahl, S. M., J. B. Allen, S. Gartner, J. M. Orenstein, M. Popovic, D. E. Chenoweth, L. O. Arthur, W. L. Farrar, and L. M. Wahl. 1989. HIV-1 and its envelope glycoprotein down-regulate chemotactic ligand receptors and chemotactic function of peripheral-blood monocytes. *J. Immunol.* 142:3553.
39. Tamma, S. M. L., N. Chirmule, H. Yagura, N. Oyaizu, V. Kalyanaraman, and S. Pahwa. 1997. CD4 cross-linking (CD4XL) induces RAS activation and tumor necrosis factor- α secretion in CD4⁺ T cells. *Blood* 90:1588.
40. DurrbaumLandmann, I., E. Kaltenhauser, H. D. Flad, and M. Ernst. 1994. HIV-1 envelope protein gp120 affects phenotype and function of monocytes in-vitro. *J. Leukocyte Biol.* 55:545.
41. Herbein, G., A. G. Doyle, L. J. Montaner, and S. Gordon. 1995. Lipopolysaccharide (LPS) down-regulates CD4 expression in primary human macrophages through induction of endogenous tumor-necrosis-factor (TNF) and IL-1- β . *Clin. Exp. Immunol.* 102:430.
42. Rigby, W. F. C., M. Waugh, and R. F. Graziano. 1990. Regulation of human monocyte HLA-DR and CD4 antigen expression, and antigen presentation by 1,25-dihydroxy vitamin-D3. *Blood* 76:189.
43. Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, H. M. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, et al. 1985. Complete nucleotide-sequence of the AIDS virus, HTLV-III. *Nature* 313:277.
44. Gurgo, C., H. G. Guo, G. Franchini, A. Aldovini, E. Collalti, K. Farrell, F. Wongstaal, R. C. Gallo, and M. S. Reitz. 1988. Envelope sequences of 2 new United-States HIV-1 isolates. *Virology* 164:531.
45. DeJong, J. J., A. Deronde, W. Keulen, M. Tersmette, and J. Goudsmit. 1992. Minimal requirements for the human-immunodeficiency-virus type-1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino-acid substitution. *J. Virol.* 66:6777.
46. Fouchier, R. A. M., M. Groenink, N. A. Kootstra, M. Tersmette, H. G. Huisman, F. Miedema, and H. Schuitemaker. 1992. Phenotype-associated sequence variation in the 3rd variable domain. *J. Virol.* 66:3183.
47. Howie, S. E. M., M. L. Fernandes, I. Heslop, T. J. Hewson, G. J. Cotton, M. J. Moore, D. Innes, R. Ramage, and D. J. Harrison. 1999. A functional, discontinuous HIV-1 gp120 C3/C4 domain-derived, branched, synthetic peptide that binds to CD4 and inhibits MIP-1 α chemokine binding. *FASEB J.* 13:503.
48. Holmes, K. L., L. M. Cuntz, and W. R. R. 1997. Cell labelling. In *Current Protocols in Cytometry*, 1st Ed. J. P. Robinson, Z. Darzynkeiwicz, P. N. Dean, L. G. Dressler, P. S. Rabinovitch, C. C. Stewart, H. J. Tanke, and L. L. Wheelless, eds. Wiley, New York, p. 4.2.1.
49. Shotton, C., C. Arnold, Q. Sattentau, J. Sodroski, and J. A. McKeating. 1995. Identification and characterization of monoclonal-antibodies specific for polymorphic antigenic determinants within the V2 region of the human-immunodeficiency-virus type-1 envelope glycoprotein. *J. Virol.* 69:222.
50. Fanci, A. S. 1995. AIDS: newer concepts in the immunopathogenic mechanisms of human-immunodeficiency-virus disease. *Proc. Assoc. Am. Physic.* 107:1.
51. Moriuchi, H., M. Moriuchi, J. Arthos, J. Hoxie, and A. S. Fauci. 1997. Promonocytic U937 subclones expressing CD4 and CXCR4 are resistant to infection with and cell-to-cell fusion by T-cell-tropic human immunodeficiency virus type 1. *J. Virol.* 71:9664.
52. Xiao, X. D., A. Kinter, C. C. Broder, and D. S. Dimitrov. 2000. Interactions of CCR5 and CXCR4 with CD4 and gp120 in human blood monocyte-derived dendritic cells. *Exp. Mol. Pathol.* 68:133.
53. Xiao, X. D., L. J. Wu, T. S. Stantchev, Y. R. Feng, S. Ugolini, H. Chen, Z. M. Shen, J. L. Riley, C. C. Broder, Q. J. Sattentau, and D. S. Dimitrov. 1999. Constitutive cell surface association between CD4 and CCR5. *Proc. Nat. Acad. Sci. USA* 96:7496.
54. Oh, S. K., W. W. Cruikshank, J. Raina, G. C. Blanchard, W. H. Adler, J. Walker, and H. Kornfeld. 1992. Identification of HIV-1 envelope glycoprotein in the serum of AIDS and ARC patients. *J. AIDS Hum. Retrovirol.* 5:251.
55. Toth, F. D., P. Mosborg-Petersen, J. Kiss, G. Aboagymathiesen, M. Zdravkovic, H. Hager, J. Aranyosi, L. Lampe, and P. Ebbesen. 1994. Antibody-dependent enhancement of HIV-1 infection in human term syncytiotrophoblast cells cultured in-vitro. *Clin. Exp. Immunol.* 96:389.